**Table 1.** General data, DNA mutations and colorectal tumors in FAP patients

| Patient | Age* (age at colectomy) | Sex | Codon | Mutation | Colorectal polyps# | Colorectal cancer |
|---|---|---|---|---|---|---|
| 1 | 18 (16) | M | 836 | TCA→TGA | ± | – |
| 2 | 24 (22) | F | 693 | AAA→TAA | +++ | + |
| 3 | 32 (31) | F | 564 | CGA→TGA | ++ | – |
| 4 | 32 (26) | M | 1061–1063 | AAACA del | ++ | – |
| 5 | 33 (27) | F | | L | + | – |
| 6 | 36 (23) | F | N | N | ++ | – |
| 7 | 37 (n) | M | 1342 | TTA→TAA | + | – |
| 8 | 39 (39) | F | | D | c | – |
| 9 | 42 (30) | M | N | N | c | + |
| 10 | 46 (19) | M | N | N | c | + |
| 11 | 46 (35) | M | 723 | T del | +++ | + |
| 12 | 46 (31) | M | n | n | c | + |
| 13 | 46 (34) | M | n | n | +++ | + |
| 14 | 47 (43) | M | 558 | A del | ± | + |
| 15 | 47 (39) | M | 159 | A insertion | ++ | – |
| 16 | 49 (27) | M | N | N | c | – |
| 17 | 49 (47) | M | N | N | ± | – |
| 18 | 49 (30) | M | 1166–1167 | TATAA del | + | – |
| 19 | 52 (26) | F | 1309–1311 | GAAAA del | c | – |
| 20 | 54 (41) | F | n | n | c | + |
| 21 | 60 (31) | M | N | N | +++ | – |
| 22 | 60 (37) | M | 367 | TC del | c | + |
| 23 | 64 (58) | F | n | n | + | + |
| 24 | 65 (60) | M | 1594–1595 | CCAG del | + | + |
| 25 | 66 (36) | M | 283 | CGA→TGA | ± | + |
| 26 | 73 (71) | M | N | N | ± | – |
| 27 | 75 (43) | F | N | N | n | – |
| 28 | 88 (85) | M | N | N | c | + |

n, not informative; N, not detected; L, large deletion from exon 6 to 15; D, duplication exon 2 + exon 3.
*Age in 2004.
#(–), no polyps/field; (±), 1–5 polyps/field; (+), 6–10 polyps/field; (++), 11–20 polyps/field; (+++), >20 polyps/field; c, confluent type.

present study, the number of male patients was twice the number of female patients, but the average TG levels exceeded 150 mg/dl between the ages of 40 and 60 years.

### Overall Prevalence of Colorectal Polyps and Cancer in FAP Patients

Among FAP patients who had >20 polyps in an endoscopic field or had polyps of the confluent type, hyperlipidemia was observed in eight patients and normal serum lipid levels in five patients (Table 1). Fifteen patients had colorectal cancer, all diagnosed as adenocarcinomas, five had gastric cancers (Patients 8, 12, 13, 16 and 21) and two had both. The percentage of hyperlipidemic patients with colorectal cancer was 53.8% (7/13) and with hypertriglyceridemia was 46.2% (6/13). Interestingly, when counting levels of serum TG ≥150 mg/dl and/or serum TC ≥220 mg/dl occurring even once as hyperlipidemic, 93.3% of the patients who had colorectal cancer demonstrated hyperlipidemic states. There were four patients who had gastric cancer with hyperlipidemia.

Statistically significant differences were not observed in serum lipid levels between the patients with colorectal cancer and those without colorectal cancer. However, the average maximum serum TG and TC values in FAP cases with colorectal cancer tended to be higher than in those without colorectal cancer, 222.7 versus 158.9 and 232.6 versus 192.5 mg/dl, respectively (Table 2 and Fig. 2).

## DISCUSSION

In the present pilot study, we found hyperlipidemia to be a relatively frequent complication in FAP patients, suggesting its possible link to colorectal cancer development. There is standard serum lipid level data for the Japanese ($n$ = 12 839, aged 4 through 99 years) collected in 36 institutes from various districts around Japan in 2000 (10). The mean serum TG and TC levels in each 10 year group were <150 and <220 mg/dl, respectively (mean TG levels for Japanese in their thirties, forties, fifties and sixties were 118, 129, 129 and 123 mg/dl; TC levels for Japanese in their thirties, forties, fifties and sixties were 195, 201, 211 and 209 mg/dl, respectively). Although males tend to have higher TG levels than females, the population ratio of hyperlipidemia did not show any difference. Thus, our pilot study suggested the need for a larger number study to confirm high TG levels in female FAP patients. Extracolonic and serious complications in FAP include adenocarcinomas in the duodenum and pancreas, and desmoid tumors developing from operation scars. Reported benign lesions are osteomas, odontomas, epidermoid cysts, stomach and thyroid adenocarcinomas, congenital hypertrophy of the retinal pigment epithelium and fundic gland polyposis (11–14), but a hyperlipidemic state has hitherto not received attention as a potentially important aspect. Three points can be raised as explanations for the lack of any focus on blood lipids: (i) myocardial infarction and stroke are not major causes of death in FAP [1.9 and 1.5% in Japanese FAP patients, respectively, ref. (15)]; (ii) hyperlipidemia may not develop at an early age [the mean ages at death of FAP patients were 44.1 years for males and 40.5 years for females before 1990, ref. (15)]; and (iii) no correlation between the *APC* gene and hyperlipidemia has hiterto been reported. Since we found only a tendency for serum TG levels to be associated with colorectal tumor development, we are now planning to investigate a large number of FAP patients for confirmation.

Prophylactic colectomy may weaken gastrointestinal function with a disorder of liver bile circulation including the lipid absorbing function of the small intestine, and if hyperlipidemia is caused by *APC* germline mutations, it is assumed that much more severe hyperlipidemia may be observed in FAP patients before prophylactic colectomy. The position of the *APC*

**Table 2.** Serum lipids levels in FAP patients

| Patient | Minimum triglyceride level (mg/dl) | Maximum triglyceride level (mg/dl) | Average triglyceride level ± SD | No. of detected TG ≥150 mg/dl/blood examinations | Maximum cholesterol level[¶] (mg/dl) |
|---|---|---|---|---|---|
| 1 | 51 | 160 | 93.7 ± 47.5 | 1/3 | 130 |
| 2 | 31 | 95 | 56.3 ± 22.8 | 0/6 | 236 |
| 3 | 43 | 129 | 82.3 ± 35.5 | 0/3 | 200 |
| 4 | 31 | 95 | 68.2 ± 22.8 | 0/6 | 271 |
| 5 | 43 | 126 | 67.2 ± 27.8 | 0/6 | 183 |
| 6 | 29 | 44 | 36.7 ± 6.0 | 0/7 | 153 |
| 7 | | | | | |
| 8 | n | n | n | | 181 |
| 9 | 40 | 148 | 67.9 ± 26.1 | 0/12 | 241 |
| 10 | 35 | 125 | 66.9 ± 21.6 | 0/34 | 220 |
| 11 | 68 | 278 | 108.2 ± 59.8 | 1/10 | 298 |
| 12 | 140 | 372 | 239.0 ± 71.4 | 9/10 | 261 |
| 13 | 124 | 425 | 236.4 ± 80.3 | 12/13 | 250 |
| 14 | 79 | 181 | 120.3 ± 43.8 | 1/3 | 228 |
| 15 | 120 | 223 | 185.3 ± 46.4 | 2/3 | 214 |
| 16 | 86 | 173 | 114.2 ± 29.7 | 2/10 | 182 |
| 17 | 133 | 214 | 173.5 ± 40.5 | 1/2 | 215 |
| 18 | 41 | 55 | 49.0 ± 5.9 | 0/3 | 185 |
| 19 | 153 | 203 | 179.0 ± 16.3 | 7/7 | 202 |
| 20 | 162 | 275 | 208.1 ± 35.0 | 7/7 | 277 |
| 21 | 167 | 429 | 274.9 ± 80.1 | 9/9 | 181 |
| 22 | 168 | 168 | 168 | 0/1 | 150 |
| 23 | 57 | 290 | 143.9 ± 78.0 | 5/14 | 237 |
| 24 | 67 | 250 | 111.8 ± 54.4 | 2/11 | 212 |
| 25 | 66 | 186 | 142.5 ± 48.3 | 2/4 | 169 |
| 26 | 72 | 215 | 138.5 ± 37.3 | 6/16 | 206 |
| 27 | 107 | 118 | 112.5 ± 5.5 | 0/2 | 265 |
| 28 | 84 | 102 | 93.6 ± 6.2 | 0/6 | 247 |

n, not informative.
[¶]Abnormally high serum levels detected more than two times are underlined.

germline mutation may affect the severity of FAP (16). The weak dominant negative effects on the wild-type APC protein by formation of unstable heterodimers result in small numbers of polyps (17). Since codons 1014–1210 and 1263–2013 are suggested to be the binding sites of β-catenin, their mutation could play an important role in formation of intestinal polyps and hyperlipidemic states (16). An FAP patient with mutations at codon 1309 (Patient 19) showed a similar hyperlipidemic state to $Apc^{1309}$ mice. Clearly, serum lipid levels may be more readily affected by environmental factors or aging than the mutated position of the *APC* gene, and other genetic factors such as *LPL* and *angiopoietin-like protein 3* may also influence the extent of hyperlipidemia (18). Therefore, our pilot study suggests the need for further studies comparing the patient before and after colectomy and also for studies to elucidate possible mechanisms linking functional genetic alteration, hyperlipidemia and colorectal cancer development.

It is of interest to point out that an agent reducing polyp formation without affecting serum lipid levels in *Apc*-deficient mice may clarify the relationship between polyps and hyperlipidemia. We are in favor of the hypothesis that hyperlipidemia is not causative at least for the onset of adenoma, but may promote intestinal polyp development. If so, an antihyperlipidemic agent is justified to be a candidate for chemoprevention. Early prophylactic colectomy is considered to be the most effective way to prevent colorectal cancer development in FAP patients, although chemopreventive agents such as selective cyclooxygenase-1 (COX-1)

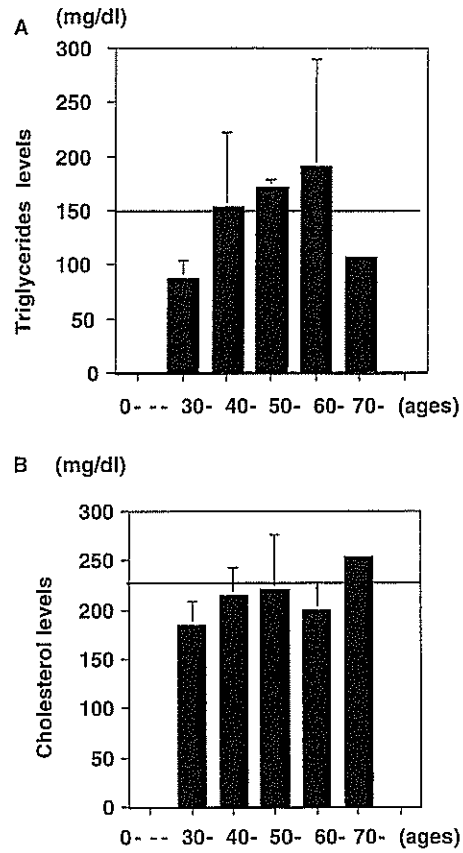


Figure 1. Serum TG (A) and TC (B) levels in FAP patients in 2002. Overall averages are arranged chronologically by age. Dotted lines are at the concentrations of 150 mg/dl for TG and 220 mg/dl for TC.

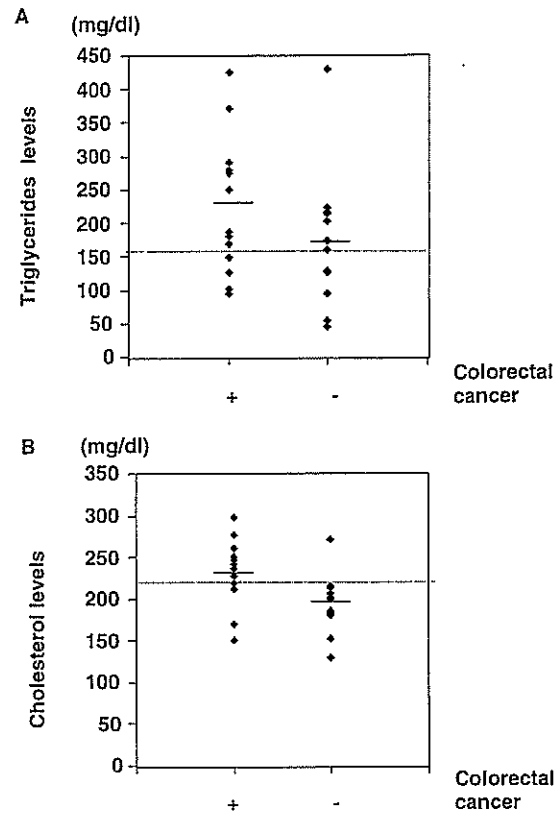


Figure 2. Serum lipid levels in FAP patients with or without a past history of colorectal cancer. The maximum serum TG (A) and TC (B) levels detected in each FAP patient from 1999 to 2004 are shown with or without past history of colorectal cancer presented as + and −, respectively. Dotted lines are at the concentrations of 150 mg/dl for TG and 220 mg/dl for TC.

and -2 inhibitors, prostaglandin receptor $EP_1$ and $EP_4$ selective antagonists, PPARα agonist and PPARγ agonist can reduce intestinal polyps in *Apc*-deficient mice (5,6,19–22). Several clinical studies have already been performed with non-steroidal anti-inflammatory drugs (NSAIDs) for prophylactic purposes in FAP patients (23,24). From our present results, not only NSAIDs and/or COX-2 inhibitors but also PPARα/γ agonists might warrant further attention and clinical trials.

In conclusion, our data lead us to hypothesize that both hyperlipidemia and polyp formation may be caused by *APC* mutation, where a hyperlipidemic state may contribute to the development of polyps. This encourages us to investigate a large number of FAP patients with matched controls. Hyperlipidemia may be observed even after prophylactic colectomy, and improving a hyperlipidemic state might be of benefit for protection against neoplasia or other adverse outcomes.

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Minireview

# Concomitant suppression of hyperlipidemia and intestinal polyp formation by increasing lipoprotein lipase activity in *Apc*-deficient mice

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## Abstract

Epidemiologically, a high-fat diet is associated with the risk of colon cancer. In addition, serum levels of triglycerides (TGs) and cholesterol have been demonstrated to be positively associated with colon carcinogenesis. We recently found that an age-dependent hyperlipidemic state (high serum TG levels) exists in *Apc*-deficient mice, an animal model for human familial adenomatous polyposis. The mRNA levels of lipoprotein lipase (LPL), which catalyzes TG hydrolysis, were shown to be downregulated in the liver and intestines of mice. Moreover, treatment with a peroxisome proliferator-activated receptor (PPAR) α agonist, bezafibrate, or a PPARγ agonist, pioglitazone, suppressed both hyperlipidemia and intestinal polyp formation in the mice, with induction of LPL mRNA. PPARα and PPARγ agonists are reported to exert antiproliferative and pro-apoptotic effects in cancer cells. One compound that also increases LPL expression levels but does not possess PPAR agnostic activity is NO-1886. When given at 400 or 800 ppm in the diet, it suppresses both hyperlipidemia and intestinal polyp formation in *Apc*-deficient mice, with elevation of LPL mRNA. In conclusion, a decrease in serum lipid levels by increasing LPL activity may contribute to a reduction in intestinal polyp formation with *Apc* deficiency. PPARα and PPARγ agonists, as well as NO-1886, could be useful as chemopreventive agents for colon cancer.

**Keywords:** *Apc*-deficient mice; hyperlipidemia; intestinal polyp; lipoprotein lipase.

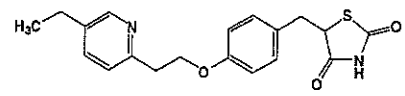
## Introduction

Colorectal cancer is one of the most common cancers in developed countries, and several epidemiological studies have suggested a correlation with obesity, a high-fat diet and hyperlipidemia (Le Marchand et al., 1997; Bruce et al., 2000), with clear links to high levels of serum triglycerides (TGs) and cholesterol (McKeown-Eyssen, 1994; Jarvinen et al., 2001). Thus, it is conceivable that not only a reduction in cholesterol levels by HMG-CoA reductase inhibitors (Agarwal et al., 1999) but also a decrease in TG levels by inhibitors may reduce colon carcinogenesis.

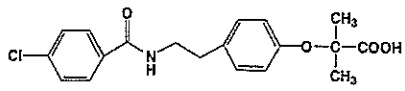
TGs are enriched in lipoproteins such as chylomicrons and very low-density lipoprotein (VLDL), and are hydrolyzed by lipoprotein lipase (LPL) to free fatty acids (FFA) and monoacylglycerol (Schoonjans et al., 1996a). LPL mRNA is expressed ubiquitously in the whole body, i.e., in adipose tissue, skeletal muscle, liver and intestine. Once synthesized, LPL is transferred to the surface of endothelial cells to become bound to membrane-anchored heparan sulfate proteoglycans (Semenkovich et al., 1989; Goldberg, 1996). Physiologically, a decrease in or deficiency of LPL is associated with hyperlipidemia (Gehrisch, 1999; Mead et al., 2002). However, no direct evidence of links between LPL and colorectal carcinogenesis has been reported.

## Age-dependent increase in TGs in *Apc* gene-deficient mice with low LPL mRNA levels

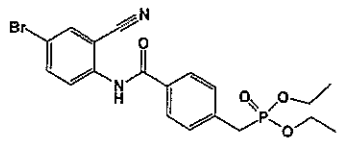
The $Apc^{1309}$ (C57BL/6J$^{Apc/Apc-1309}$) mouse, an animal model of human familial adenomatous polyposis (FAP), develops large numbers of intestinal polyps because of a truncation mutation in the *adenomatous polyposis coli* (*Apc*) gene ($Apc^{1309}$; Quesada et al., 1998). It is considered to have advantages for evaluation of chemopreventive agents, as with other FAP model mice, such as $Apc^{Min}$ (Min), $Apc^{\Delta 716}$ and $Apc^{1638}$ mice (Moser et al., 1990; Fodde et al., 1994; Oshima et al., 1995). During experiments to evaluate chemopreventive agents, we found that the serum of the $Apc^{1309}$ mouse is very pale in color and compared lipid levels with similarly aged wild-type mice. Although no significant differences were observed at 6 weeks of age, TG levels were obviously increased in $Apc^{1309}$ mice with aging; the average value at 12 weeks was almost 10-fold higher than that at 6 weeks (Niho et al., 2003a). Such an increase was not observed in the wild-type counterparts. Total cholesterol levels in $Apc^{1309}$ mice were also increased between 6 and 12 weeks of age, from 87.0±3.2 to 162.4±33.0 mg/dl. Moreover, FFA levels were increased with age. Differences in serum lipid levels were not observed between male and female $Apc^{1309}$ mice aged 12 weeks. An age-dependent hyperlipidemic state was also observed in Min mice (Niho et al., 2003a). TG levels in female Min mice at 15 weeks of age were elevated to levels almost 10-fold higher than those at 8 weeks of age. Values for total cholesterol at 8 and 15 weeks of age were 83.7±6.3 and 107.8±15.6 mg/dl, and those for FFA were 1.0±0.1 and 3.1±0.4 mEQ/l, respectively.



(±)-5-[4-[2-(5-Ethyl-2-pyridyl)ethoxy]benzyl]-thiazolidine-2,4-dione monohydrochloride (Pioglitazone)



2-[4-(2-[4-Chlorobenzamido]ethyl)phenoxy]-2-methylpropanoic acid (Bezafibrate)



4-[(4-Bromo-2-cyanophenyl)carbamoyl]benzylphosphonate (NO-1886)

**Figure 1** Structures of LPL inducers.

To cast light on mechanisms underlying the increased levels of serum lipids, especially TGs, in *Apc*-deficient mice with age, we investigated expression levels of mRNAs encoding metabolic enzymes (Niho et al., 2003a). In the liver and small intestine of *Apc*$^{1309}$ and wild-type mice at 6, 8 and 12 weeks of age, there were no obvious differences in mRNA levels for fatty acid synthase (FAS), stearoyl-CoA desaturase-1, acyl-CoA oxidase, carnitine palmitoyl transferase-1 and phosphoenolpyruvate carboxykinase, enzymes involved in the hydrolysis of TGs, lipogenesis, β-oxidation and glucose homeostasis. Similar expression levels for these genes were also observed in Min and wild-type mice at all ages. However, LPL mRNA levels in the liver were clearly lowered in *Apc*$^{1309}$ mice at 6, 8 and 12 weeks, and in Min mice at 8, 11 and 15 weeks, proportional to aging. A similar decrease was also observed for small intestinal mRNA levels. These data provide evidence that the expression levels of LPL, which catalyzes TG hydrolysis, correlate with hypertriglyceridemia in *Apc*$^{1309}$ and Min mice.

## Reduction in serum TG levels in *Apc*-deficient mice by administration of the PPARγ ligand, pioglitazone, or the PPARα ligand, bezafibrate

Pioglitazone, {(±)-5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]thiazolidine-2,4-dione monohydrochloride}, is a potent PPARα ligand that also demonstrates weak binding to PPARγ; bezafibrate, {2-[4-(2-[4-chlorobenzamido] ethyl)phenoxy]-2-methylpropanoic acid}, is a specific PPARα ligand (Sakamato et al., 2000) (Figure 1). PPARs are predominantly expressed in the liver, heart, kidney, intestinal mucosa and brown adipose tissue, all with high catabolic rates for fatty acids and peroxisomal metabolism (Schoonjans et al., 1996b). Thus, these ligands are used clinically to improve hypertriglyceridemia and hypercholesterolemia through induction of lipid metabolism-related genes such as *LPL* (Schoonjans et al., 1996a). Transcriptional regulation of the *LPL* gene has been reported to be mediated through binding of PPAR-retinoid X receptor heterodimers to the functional responsible element sequence in the *LPL* gene promoter lesion (Schoonjans et al., 1996a).

Administration of each ligand for 6 weeks did not affect food intake or behavior of male *Apc*$^{1309}$ mice. However, final body weights in the groups treated with 100 and 200 ppm pioglitazone were increased to 113–115% of those in the AIN-76A basal diet group, and those in the bezafibrate-treated groups increased to 118–122% (Niho et al., 2003a). Serum lipid levels are summarized in Table 1. Serum TG levels at 12 weeks of age were reduced by 44% and 50% by 100 and 200 ppm pioglitazone, respectively. The respective levels of total cholesterol were also decreased by 15% and 28%. Administration of pioglitazone caused a 27% decrease in FFA levels at both concentrations, but significance was not attained. Similar results were obtained in male Min mice (Niho et al., 2003b). Serum TG levels in the basal diet group were elevated 13–15-fold relative to those in wild-type counterparts at 20 weeks of age. They were reduced dose-dependently by treatment with 100, 200, 400 and 1600 ppm pioglitazone from 6 to 20 weeks of age, with suppression to almost the wild-type level at 1600 ppm (Niho et al., 2003b). Although the values for total cholesterol in Min mice were not changed by pioglitazone treatment, the balance of HDL-C, LDL-C, and VLDL-C in the total cholesterol of Min mice was improved to almost the wild-type level. The levels of FFA in Min mice were decreased by pioglitazone to 44% of the untreated control value.

Administration of bezafibrate to *Apc*$^{1309}$ mice reduced serum TG levels dose-dependently by 30% and 55% ($p<0.05$) at 100 and 200 ppm, respectively (Niho et al., 2003a). The levels of total cholesterol and FFA showed a tendency to decrease by 6–18%.

## Suppression of intestinal polyp formation in *Apc*-deficient mice by pioglitazone or bezafibrate

In *Apc*-deficient mice, almost all polyps developed in the small intestine, with only a few in the colon (Niho et al., 2003a,b, 2005). The total numbers of polyps in the groups treated with pioglitazone at 100 and 200 ppm in *Apc*$^{1309}$ mice were reduced to 67% ($p<0.05$) of the value in the untreated control group (Table 1). The numbers of polyps in the proximal and middle parts of the small intestine in *Apc*$^{1309}$ mice treated with 200 ppm pioglitazone were 58% ($p<0.05$) and 61% ($p<0.01$) of the untreated control values, respectively. Dietary administration of 100 and 200 ppm bezafibrate reduced the total numbers of polyps by 13% and 25% ($p<0.05$), respectively (Table 1). The numbers of polyps in the proximal, middle, and distal parts of the small intestine in *Apc*$^{1309}$ mice treated with 100 and 200 ppm bezafibrate were also reduced by 4–27%, albeit without statistical significance.

The size distribution of intestinal polyps in groups on a basal diet and treated with pioglitazone or bezafibrate was also investigated (Niho et al., 2003a,b). Treatment of *Apc*$^{1309}$ mice with 100 and 200 ppm pioglitazone reduced the numbers of polyps measuring more than 1.0 and more than 0.5 mm in diameter, respectively. On the other

**Table 1** Serum lipid levels and total number of polyps in *Apc*-deficient mice.

| Mice (age) | Agent | Dose (ppm) | Triglycerides (mg/dl) | Cholesterol (mg/dl) | Total number of polyps/mouse |
|---|---|---|---|---|---|
| *Apc*$^{1309}$ (12 weeks) | Pioglitazone | 0 | 710±131 | 133±15 | 36.7±2.7 |
| | | 100 | 396±116 | 113±15 | 24.6±4.4 |
| | | 200 | 355±101 | 96±15 | 24.5±4.2 |
| | Bezafibrate | 0 | 682±119 | 141±17 | 37.7±2.9 |
| | | 100 | 480±111 | 132±10 | 32.7±1.7 |
| | | 200 | 309±70 | 119±14 | 28.3±2.7 |
| Min (20 weeks) | Pioglitazone | 0 | 512±127 | 113±11 | 71.9±6.7 |
| | | 100 | 324±117 | 113±12 | 45.5±10.2 |
| | | 200 | 171±69 | 104±8 | 32.7±11.3 |
| | | 400 | 86±39 | 104±14 | 33.5±19.0 |
| | | 1600 | 21±3 | 121±9 | 6.2±5.1 |
| | NO-1886 | 0 | 607±120 | 147±13 | 121.7±26.0 |
| | | 400 | 238±49 | 101±9 | 58.0±10.8 |
| | | 800 | 186±81 | 113±10 | 50.5±7.8 |

Data are mean±SEM.

hand, 100 and 200 ppm bezafibrate reduced the numbers of polyps, especially those of 0.5–1.5 mm in diameter. Min mice given 100–1600 ppm pioglitazone for 14 weeks demonstrated decreased numbers of intestinal polyps to 9–63% of the control value (Table 1). The numbers of polyps in the proximal, middle, and distal parts in Min mice treated with 200 ppm pioglitazone were reduced by 50–60%, with particularly marked effects on polyps measuring less than 1.0 and 3.0–4.0 mm in diameter.

Administration of 100 and 200 ppm pioglitazone or bezafibrate raised hepatic LPL mRNA levels in *Apc*$^{1309}$ mice. Similar upregulation was also evident for small intestinal mRNA levels, although the degree of elevation was small.

## Concomitant suppression of serum lipid levels and intestinal polyp formation in Min mice by a LPL selective inducer, NO-1886

It is well known that PPARγ and PPARα agonists induce cell growth arrest and apoptosis in various types of cancer cells, including colon cancer cells (Rosen and Spiegelman, 2001). Thus, the decreases in polyp numbers in *Apc*$^{1309}$ and Min mice by pioglitazone or bezafibrate in our study might have resulted from such actions. LPL selective inducers are necessary for determining the relationship between hyperlipidemia and intestinal carcinogenesis, and NO-1886, 4-[(4-bromo-2-cyanophenyl)carbamoyl]benzylphosphonate, chemically synthesized at Otsuka Pharmaceutical Factory (Tsutsumi et al., 1993) (Figure 1), is thus a useful tool. Using a reporter gene assay, NO-1886 was revealed not to possess PPARγ and PPARα agonistic activity, unlike bezafibrate and pioglitazone (Doi et al., 2003). In the next study, we therefore examined the effects of 400 and 800 ppm NO-1886 in the diet on both hyperlipidemia and intestinal polyp formation in female Min mice (Niho et al., 2005).

Administration for 13 weeks did not affect body weights or clinical signs of Min mice throughout the experimental period and amounts of daily food intake did not differ among the groups. In addition, there were no changes observed in any organ weights that could be attributable to toxicity. Administration of 400 and 800 ppm NO-1886 clearly decreased serum TG levels to 39% and 31% of the untreated control value, respectively. The levels of total cholesterol were also decreased by 31% and 23%. Moreover, levels of both TG-rich lipoproteins, VLDL-C and LDL-C, were dramatically decreased by NO-1886 treatment to 15% and 32% of the untreated control values, respectively. In contrast, HDL-C levels were increased to the wild-type value at 800 ppm. Overall, administration of NO-1886 improved the balance of HDL-C, LDL-C, and VLDL-C in the total cholesterol of Min mice. LPL mRNA levels in the liver and the small intestine were markedly increased by treatment with NO-1886.

The data for numbers of intestinal polyps in the AIN-76A basal diet and NO-1886-treated groups are also shown in Table 1 (Niho et al., 2005). The total number of polyps were significantly decreased by administration of 400 and 800 ppm NO-1886 to 48% and 42% of the untreated control value, respectively, with reduction in the proximal, middle, and distal parts by 63%, 57% and 45% with 400 ppm, and by 74%, 63% and 49% with 800 ppm. Treatment with NO-1886 also significantly decreased the numbers of colon polyps. Administration of NO-1886 reduced the numbers of polyps of all sizes (0.5–3.0 mm in diameter) observed in the basal diet groups.

## Down-regulation of COX-2 expression levels by NO-1886

To elucidate the mechanisms of the NO-1886 effects on colon carcinogenesis, we also investigated expression levels of mRNAs for inflammation-associated enzymes, cyclooxygenase-1 (COX-1), COX-2 and inducible nitric oxide synthase (iNOS), in DLD-1 human colon cancer cells (Niho et al., 2005). RT-PCR analysis revealed that the TGFα-stimulated COX-2 mRNA levels were reduced to non-stimulated levels by NO-1886 at 5 and 10 μM. On the other hand, there was no obvious change in mRNA levels for COX-1 and iNOS. The results were also confirmed by a β-gal reporter gene assay in DLD-1 cells. COX-2 promoter transcriptional activity was normalized

to total protein as measured by colorimetric assay. Treatment of cells with 100 ng/ml TGFα for 48 h increased COX-2 promoter transcriptional activity to 1.6-fold of the control value, whereas NO-1886 at 5 and 10 μM suppressed TGFα-stimulated COX-2 promoter transcriptional activity to only 1.2-fold of the control value, with no significant cytotoxicity. Consistent with the *in vitro* data, administration of NO-1886 at 400 and 800 ppm reduced mRNA levels of COX-2 in normal parts of the small intestine of Min mice at 20 weeks of age.

It is well known that expression of COX-2 is markedly elevated in colon cancers of humans and AOM-treated rats and in intestinal polyps of *Apc*-deficient mice (Sano et al., 1995; DuBois et al., 1996; Williams et al., 1996), playing an important role in cancer cell proliferation and angiogenesis (Tsujii et al., 1998). Therefore, suppression of COX-2 by NO-1886 is one possible mechanism underlying the suppression of intestinal polyp development.

## Conclusions

Our studies have demonstrated that a hyperlipidemic state exists in two strains of FAP model mice. The levels of serum lipids, especially TGs, are thus dramatically increased with age in both $Apc^{1309}$ and Min mice. Possible involvement of hyperlipidemia in human FAP and in sporadic colorectal tumor patients is now under investigation.

It is interesting that LPL mRNA levels in the livers and small intestines of *Apc*-deficient mice were markedly lower than those of wild-type mice. At present, the biological relationship between *Apc* deficiency and severe hyperlipidemia is uncertain, but it has been reported that Wnt signaling inhibits the transcription factors CCAAT/enhancer binding protein and PPAR, and maintains preadipocytes in an undifferentiated state (Ross et al., 2000), suggesting the involvement of Wnt signaling in lipogenesis.

Although it still cannot be stated with certainty whether hyperlipidemia is a leading cause of intestinal polyp formation, our study demonstrated that LPL inducers, such as the PPAR ligands pioglitazone and bezafibrate, and NO-1886 have the potential to suppress both hyperlipidemia and polyp formation in *Apc*-deficient mice. It is therefore speculated that LPL activity itself may play an important causative role in tumor induction.

A large number of chemopreventive agents have been examined in light of their effects on colon carcinogenesis in *Apc*-deficient mice models, including enzyme inhibitors (ornithine decarboxylase and iNOS), non-steroidal anti-inflammatory drugs, micronutrients (selenium, vitamins, etc.) and PPAR activators (Jackson et al., 2003). LPL inducers have an advantage as novel targeting chemopreventive reagents and could be useful as new strategies. In addition, PPAR agonists in chemoprevention show a limitation in comparison with the above-mentioned agents because of their controversial effect on colon carcinogenesis. Previous animal studies have suggested that activation of PPARα reduces intestinal polyp formation (Lefebvre et al., 1998), whereas PPARβ and PPARγ activation by specific agonists results in either increased (Tanaka et al., 2001) or reduced formation of neoplastic lesions (Corpet and Pierre, 2003). We demonstrated that both PPARα and PPARγ agonists suppress intestinal polyp formation. The number of polyps was reduced to a greater extent by pioglitazone than by bezafibrate. Moreover, pioglitazone reduced the number of polyps of all sizes, whereas bezafibrate reduced only small polyp numbers. It can be speculated that the differential effects of PPAR subtype specific agonists may be associated with their effects on lipid metabolism and may also be affected by their chemical structure. For instance, troglitazone, which has a quinone structure, causes lethal liver toxicity and may increase polyp formation (Tettey et al., 2001).

Clearly, we need to elucidate the mechanisms underlying the hypertriglyceridemia in FAP model mice and the roles of LPL in intestinal polyp development, in which COX-2 suppression may be partly involved. For the present, however, we can conclude that PPAR ligands and NO-1886 could be useful as chemopreventive agents for colon cancer.

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# Roles of Prostanoids in Colon Carcinogenesis and their Potential Targeting for Cancer Chemoprevention

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**Abstract:** Prostanoids are produced in response to numerous growth factors and environmental stimuli. Their synthesis is dependent on two cyclooxygenase (COX) enzymes, COX-1 and COX-2, which are rate-limiting for the production of prostaglandins (PGs) and thromboxanes from free arachidonic acid. Selective inhibitors of both COX forms have the potential to inhibit colon tumorigenesis, and there is abundant documented evidence of elevated expression of COX-2 in colon tumors and a variety of other malignancies. The resultant high level $PGE_2$ production may play an important role in cell proliferation, modulation of apoptosis, angiogenesis, inflammation and immune surveillance. Prostanoids exert their biological actions through binding to eight specific membrane receptors; the four subtypes $EP_1$ to $EP_4$ for $PGE_2$; DP for $PGD_2$; FP for $PGF_2$; IP for $PGI_2$; and TP for thromboxane $A_2$. Recently, genetic and pharmacologic experiments have suggested that all $PGE_2$ receptors can contribute to colon tumorigenesis. Moreover, it is suggested that $EP_1$ and $EP_4$ play roles in polyp formation independently. It is important to determine details of the down-stream signaling pathways of prostanoid receptors for further understanding of the mechanisms of cancer development. Furthermore, it is anticipated that development of specific receptor antagonists will provide new advantageous tools for chemoprevention.

Key Words: Chemoprevention, colon carcinogenesis, EP receptors, Prostanoids.

## EPIDEMIOLOGICAL FINDINGS AND CLINICAL EVIDENCE OF COLON CANCER CHEMOPREVENTION

### A) Epidemiological Findings

Several epidemiological studies have indicated that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of colon cancer. For example, it has been reported that individuals taking aspirin demonstrate at most 40% reduction in the relative risk of colorectal cancer and associated mortality [1]. Although the molecular mechanisms by which NSAIDs reduce colorectal cancer and other neoplasms remain to be determined in detail, the most likely possibility is due to their inhibition of cyclooxygenase (COX).

COX involvement in prostanoid synthesis is schematically illustrated in Fig. (1). PGs are synthesized by human tissues ubiquitously and are involved in diverse biological processes such as blood clotting, maintaining blood vessel tone, bone metabolism, immune responses, implantation, ovulation, initiation of labor, kidney function, nerve growth, inflammation and wound healing [2]. The release of arachidonic acid (AA), a 20-carbon polyunsaturated fatty acid, from cell membrane phospholipids is mainly mediated *via* the action of phospholipase $A_2$ ($PLA_2$) [3]. COX catalyzes the conversion of AA to $PGG_2$ and $PGH_2$. Addition of molecular oxygen produces the unstable product, $PGG_2$, which is rapidly converted to $PGH_2$ by the peroxidase activity of the enzyme. It is well established that there are two isoforms of COX, the constitutive enzyme COX-1, present in many cells and tissues, and the inducible enzyme, COX-2, produced in response to growth factors, mitogens, pro-inflammatory cytokines and mucins [4, 5]. $PGH_2$ is additionally isomerized to $PGE_2$ by $PGE_2$ synthase [6] and converted to a variety of other PGs such as $PGD_2$, $PGF_2$, $PGI_2$, and thromboxane $A_2$ ($TXA_2$) by their respective PG synthases. Nonenzymatic dehydration of $PGD_2$ results in generation of $PGJ_2$, 12-$PGJ_2$, and 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ (15-$\Delta$-$PGJ_2$) [7].

### B) Clinical Evidence

Enhanced COX-2 expression and increased $PLA_2$ activity have been observed in human colon cancer tissues and pre-malignant polyps compared with the non-lesional and/or normal colon tissues, localized in epithelial cancer cells, inflammatory cells, vascular endothelium, and fibroblasts [8-11]. Furthermore, the major prostanoid found in colorectal cancers appears to be $PGE_2$ [12]. A COX-2 selective inhibitor, celecoxib, and conventional NSAIDs which inhibit both COX-1 and COX2, indomethacin and sulindac, (Fig. (2)) have actually caused regression of existing colorectal polyps in patients with familial adenomatous polyposis (FAP) [13-15]. FAP patients, affected by a rare hereditary condition resulting from germline inactivation of one allele of the *adenomatous polyposis coli (APC)* gene, develop tens to thousands of adenomatous polyps. Recently, it was found that sporadic colorectal cancers also acquire somatic mutations in the *APC* gene with defects in APC-dependent signaling [16].

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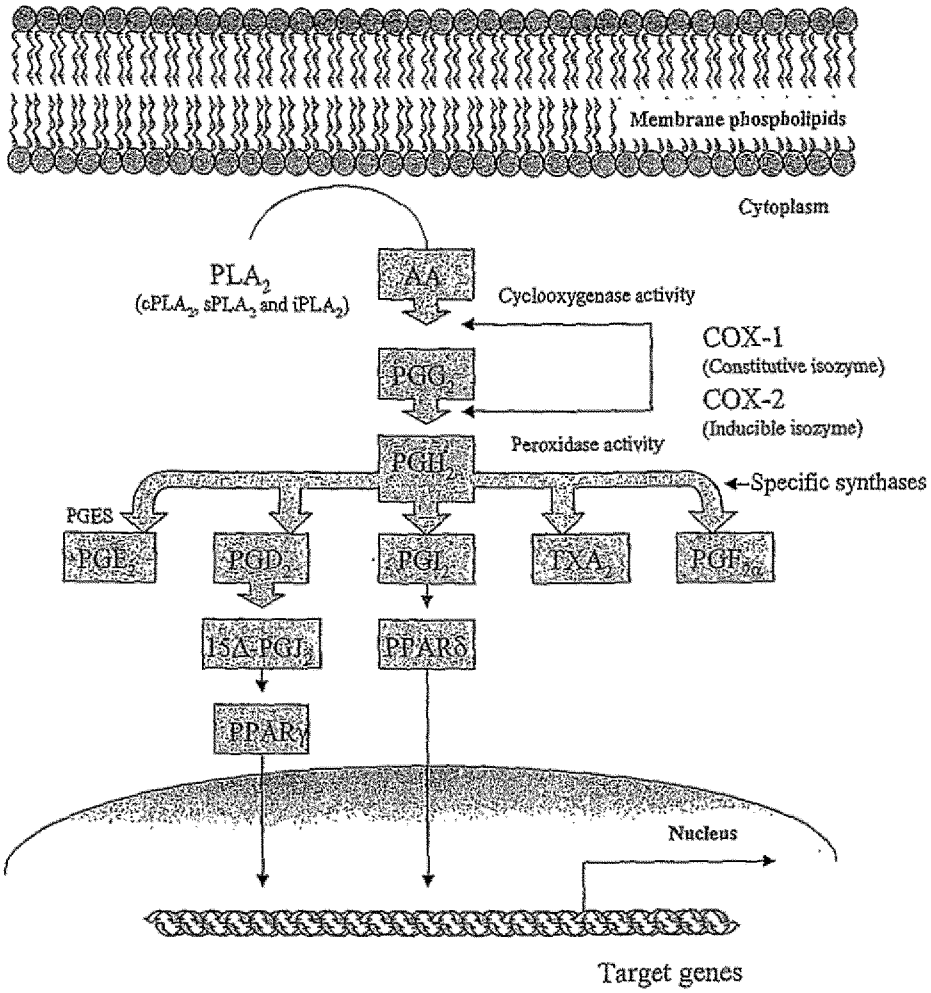



**Fig. (1).** Schematic illustration of the pathways involved in prostanoid synthesis. AA = Arachidonic acid; COX = Cyclooxygenase; PG = Prostaglandin; PGES = $PGE_2$ synthase; $PLA_2$ = Phospholipase $A_2$; PPAR = peroxisome proliferator-activated receptor; $TXA_2$ = Thromboxane $A_2$.

## CYCLOOXYGENASE INVOLVEMENT IN COLON CARCINOGENESIS

Conventional NSAIDs, including indomethacin, sulindac and aspirin have been shown to inhibit the development of colon cancers in animal models [17-19]. Treatment with COX-2 selective inhibitors alone or combined with COX-2 gene knockout results in reduction of polyp development in *Apc*$^{\Delta 716}$ mice [20, 21] and there is abundant evidence from animal models that COX-2 plays an important role in colon carcinogenesis. In addition, both pharmacologic and genetic studies have indicated that COX-1 also makes a key contribution to intestinal tumorigenesis. Dietary administration of 1200 ppm mofezolac, a COX-1 selective inhibitor (Fig. (2)), was thus found to reduce the number of aberrant crypt foci (ACFs), putative preneoplastic lesions, per rat, and the 5-bromodeoxyuridine (BrdU) labeling index of the crypt epithelium. Treatment with the same dose of mofezolac reduced the number of intestinal polyps in APC1309 mice to 59% of that in the control diet group [22]. Homologous genetic disruption of either COX-1 or COX-2 furthermore markedly reduced polyp formation in Min mice with a nonsense mutation in the *APC* gene [23]. Combined treatment with 600 ppm mofezolac and 400 ppm nimesulide, a COX-2 selective inhibitor (Fig. (2)), in the APC1309 female mice resulted in inhibition of polyp development that was almost equal to the sum of the effects of each agent alone [24]. The results indicate that COX-1 and -2 may contribute to polyp formation independently and support the idea that prostanoid production by either of the COX isoforms plays an important role in colon carcinogenesis. Structures of some COX-1 and COX-2 selective inhibitors are shown in Fig. (2).

## ROLE OF PROSTAGLANDIN RECEPTORS

### A) Effect of $PGE_2$ Receptor Deficiency and Treatment with Antagonists

We have conducted a series of experiments to determine effects of prostanoid receptors on colon carcinogenesis with a genetic approach. Examination of the induction of ACFs by AOM in knockout mice deficient in $EP_1$, $EP_2$, $EP_3$, $EP_4$, DP,

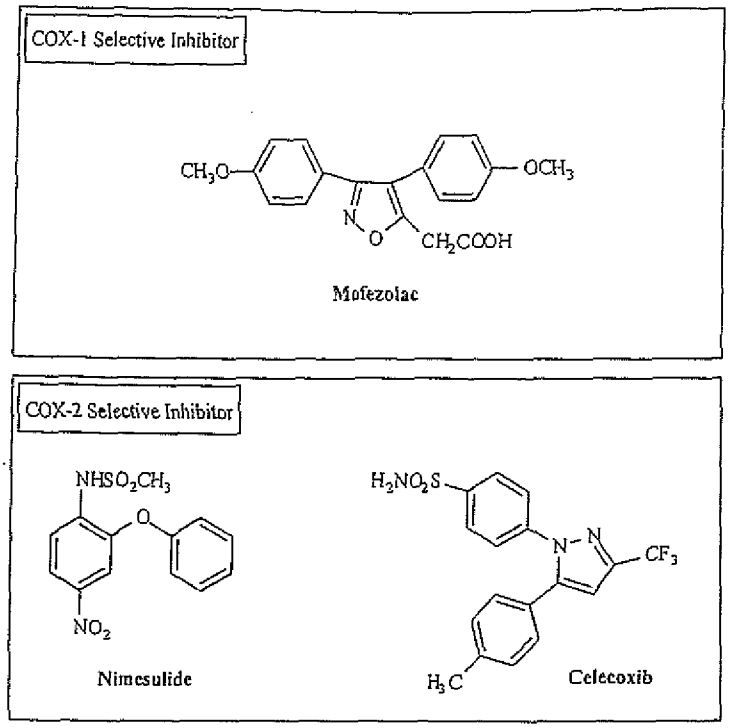



Fig. (2). Structures of COX-1 and COX-2 inhibiors.

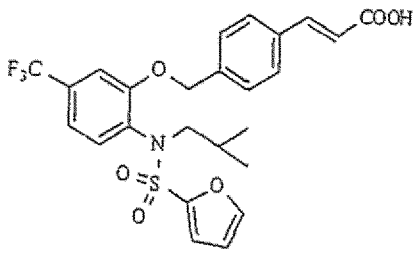
FP, IP or TP, revealed decrease only in the $EP_1$ and $EP_4$-knockout cases, to approximately 60% and 56% of the level in wild-type mice, respectively [25, 26]. A pharmacological approach with selective antagonists for $EP_1$ and $EP_4$ receptors was then adopted to confirm involvement of the two receptors in colon carcinogenesis using two animal models, the AOM-induced ACF model and the Min mouse model. Structures of the $EP_1$ receptor selective antagonists, ONO-8711 and ONO-8713, and the $EP_4$ receptor selective antagonist, ONO-AE2-227, are shown in Fig. (3). Both ONO-8711 and ONO-8713 inhibited development of AOM-induced ACFs in male C57BL/6J mice. Moreover, when Min mice were given 500 ppm ONO-8711 in the diet, the number of intestinal polyps was significantly reduced to 57% of that in the basal diet group [25]. Administration of ONO-AE2-227 to AOM-treated wild mice and Min mice decreased ACFs and intestinal polyp formation, respectively. Interestingly, in the latter case the number of polyps $\geq$1.0 mm in diameter, but not those <1.0 mm in diameter, were reduced, suggesting reduction in tumor growth [26]. In order to determine the contribution of $EP_1$ and $EP_4$ receptors to intestinal tumorigenesis, further experiments were designed to investigate the combined effects of $EP_1$ and $EP_4$ antagonists, ONO-8711 and ONO-AE2-227, on polyp formation in APC1309 mice. A summative tendency for suppression was also observed with respect to the size and numbers of polyps in the intestine. In this experiment, polyp size reduction was more remarkable with the $EP_4$ antagonist, while reduction in the number was more pronounced with the $EP_1$ antagonist [27].

Regarding the other receptor types, it was reported that homozygous deletion of the $EP_2$ receptor gene also resulted in decrease of intestinal polyp formation in the APC knockout mice [28]. Moreover, $EP_3$ appears to play an opposing important role in protecting the colon from tumor development induced by AOM [29]. Long-term colon carcinogenesis experiments with $EP_1$, $EP_2$ and $EP_4$ antagonists are now needed to decide which are significant with respect to involvement in cancer progression.

### B) Effect of $PGE_2$ Treatment

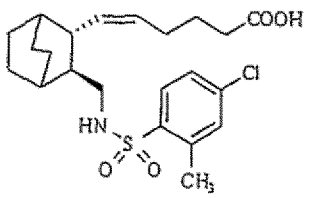
It is known that the $PGE_2$ levels are elevated in the colon tumor as compared with surrounding normal tissue [12]. Intraperitoneal injection of 7.7 μg $PGE_2$ once a week for 25 weeks significantly increased the AOM-induced rat colon tumor incidence (percentages rats with tumors, 92 versus 53), especially for adenocarcinomas (92 versus 47%), and multiplicity (number of tumors per rat, 2.8 versus 1.0) in comparison with animals treated with the vehicle alone [30]. There are reports suggesting that $PGE_2$ is important in the maintenance of tumor integrity and may be adequate to promote colon cancer development. Administration of the $PGE_2$ analogues 16,16-dimethyl-$PGE_2$ and 17-phenyl-trinor-$PGE_2$ of 10 μg each 3 times daily *via* gavage or intraperitoneal injection to Min mice counteracted the reduction in number of polyps caused by NSAIDs (piroxicam and sulindac) treatment, while elevating the intracellular $Ca^{2+}$ concentration [31]. However, not all the data are consistent and Min mice treated with a stable $PGE_2$ analogue 16,16-dimethyl-$PGE_2$ from 6-18 weeks of age demonstrated an approximately 50-70% decrease in tumor incidence, with a 20-50% reduction in the number of lesions and a 10-70% reduction in their size [32].

ONO-8713



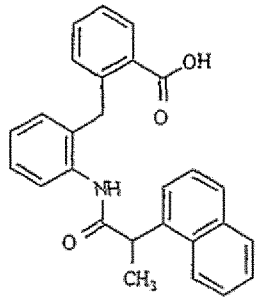
6-[(2*S*,3*S*)-3-(4-Chloro-2-methylphenylsulfonylaminomethyl)-bicyclo[2.2.2]octan-2-yl]-5*Z*-hexenoic acid

ONO-8711



4-[2-[*N*-Isobutyl-*N*-(2-furylsulfonyl)amino]-5-trifluoromethylphenoxymethl]cinnamic acid

ONO-AE2-227



2-[2-{2-(1-Naphthyl)propanoylamino}phenyl] methylbenzoic acid

Fig. (3). Structures of $EP_1$ and $EP_4$ antagonist.

## DOWNSTREAM SIGNALING PATHWAYS FROM ACTIVATED PROSTANOID RECEPTORS

Two classes of prostanoid receptors exist which can transduce signals from prostanoids and other ligands: the G protein-coupled cytoplasmic receptor class [33] and the nuclear peroxisome proliferator-activated receptor (PPAR) class [34]. As noted above, the prostanoids such as $PGE_2$, $PGD_2$, $PGF_2$, $PGI_2$ and $TXA_2$ exert their biological actions through binding to the eight specific membrane receptors; the four subtypes $EP_1$ to $EP_4$ for $PGE_2$; DP for $PGD_2$; FP for $PGF_2$; IP for $PGI_2$; and TP for $TXA_2$ [35, 36]. 15-Δ-$PGJ_2$ and $PGI_2$ are the ligands of PPARγ and PPARδ, respectively (Fig. (4)).

### A) $EP_1$ and $EP_1$ Variants

The $EP_1$ receptor is a transmembrane G protein-coupled receptor, similar to other $PGE_2$ receptors, and its rat cDNA clone encodes 405 amino acid residues with seven transmembrane-spanning domains. $EP_1$ signals transmitted by increased intracellular $Ca^{2+}$ concentrations are known to activate protein kinase C (PKC) [35, 36] but the actual signal transduction mechanisms are not known in detail.

There are multiple receptor isoforms of $EP_1$, $EP_3$, FP and TP, modified by RNA splicing. The rat $EP_1$-variant receptor is translated from mRNA which is not spliced at nucleotide position 952 in the segment VI transmembrane region and retains the ligand binding activity with affinity and specificity similar to rat $EP_1$ receptor, but without the ability to couple with signal transduction systems by itself. When rat $EP_1$-variant receptor was stably co-expressed with rat $EP_1$ or rat $EP_4$ receptor in CHO cells, the $Ca^{2+}$ mobilization mediated by the $EP_1$ receptor and the cAMP formation due to activation of the endogenous $EP_4$ receptor were significantly suppressed [37].

### B) $EP_2$ and $EP_4$

Activation of $EP_2$ and $EP_4$ receptors in both cases involves coupling with stimulatory G protein (Gs protein), leading to up-regulation of adenylate cyclase (AC) activity. In the AC pathway, increased cAMP levels result in activation of cAMP-dependent protein kinase (PKA) and increase in a transcriptional factor that binds to cAMP-responsive elements (CRE), transactivating the transcription of specific primary response genes [37]. However there are clear differences between $EP_2$ and $EP_4$ in their structure and functions. cDNAs encoding $EP_2$ and $EP_4$ receptors share less than 30% amino acid homology and are no more related to each other than to other prostanoid receptor subtypes [38]. In fact, the $EP_2$ receptor shows a closer phylogenetic relationship to the DP and IP receptors than it does to the $EP_4$ receptor. Largely due to differences in the carboxyl (C)-terminal domain, the human $EP_4$ receptor is considerably larger than the human $EP_2$ receptor (488 versus 358 amino acids).

Both $EP_2$ and $EP_4$ receptors can activate T-cell factor (Tcf) signaling (Fig. (4)). However, $EP_2$ receptors do this primarily through a PKA-dependent pathway, whereas $EP_4$ receptors primarily utilize a phosphatidylinositol 3-kinase (PI3K)-dependent pathway [39]. $PGE_2$ stimulation of $EP_4$, but not $EP_2$, leads to phosphorylation of the extracellular signal-regulated kinases (ERKs) through a PI3K-dependent mechanism [40]. $EP_2$ is responsible for inducing the COX-2 gene through a positive feedback mechanism by $PGE_2$ [28] because of CRE stimulation in its promoter region [41].

### C) $EP_3$ and $EP_3$ Variants

The activated $EP_3$ receptor couples with Gi protein, leading to inhibition of AC activity and resulting in a decrease of cAMP concentration. Known to have multiple splice variants in the human (9 variants), mouse (3 variants), rat (4 variants) and rabbit (4 variants), in the rat case, the $EP_3$ splice variants differ in the sequence of the intracellular C-terminus [42,43]. Interestingly, rabbit $EP_3$ receptor splice variants can stimulate CRE/β-galactosidase-mediated activity, and this appears to be independent of cAMP generation [44].

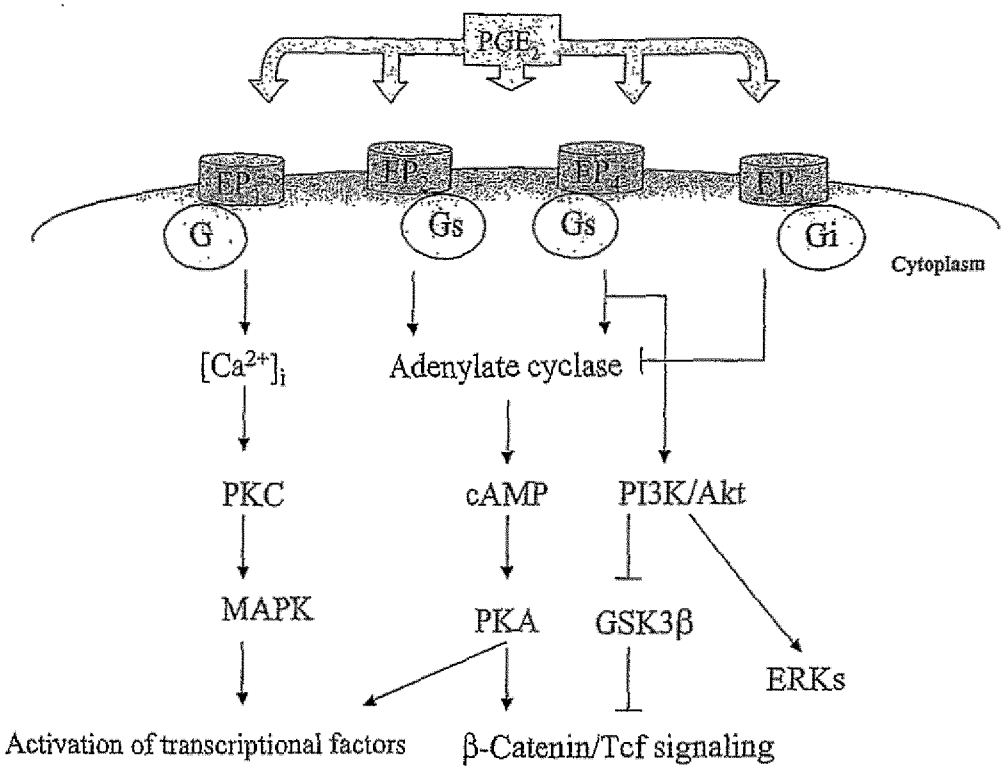



Fig. (4). Down stream signaling pathways from $PGE_2$. ERK=Extracellular signal-regulated kinase; GSK=glycogen synthase kinase-3; MAPK=Mitogen-activated protein kinase; PKC=Protein kinase C; PI3K=Phosphatidylinositol 3-kinase.

## MECHANISMS BY WHICH PROSTANOIDS CONTRIBUTE TO CARCINOGENESIS

Many lines of evidence suggest that modulation of prostanoid synthesis and its functions are promising targets for the prevention or treatment of colon cancer [45]. Although the question of how prostanoids promote colon carcinogenesis has not been fully elucidated to date, several mechanisms can be speculated.

### A) Cell Proliferation

$PGE_2$ may stimulate cell proliferation by production of growth factor [46, 47], activation of PI3K/Akt signaling and activation of Src, a downstream effector of the epithelial growth factor (EGF) receptor [48]. This involves activation of transcriptional factors such as c-fos, an induced response gene, and the early growth response factor-1 (EGR-1). The PI3K/Akt pathway promotes growth factor-mediated cell survival, inhibits apoptosis [49] and modulations cell cycle progression *via* modifications to cyclin D1 and $p27^{kip1}$ [50, 51].

Treatment of LS-174 human colon cancer cells, seeded in Matrigel®, with $PGE_2$ resulted in a dose-dependent increase in colony diameter through actions on the $EP_4$ receptor [52]. Consistent with this, an $EP_4$-selective agonist, 16-(3-methoxymethyl)phenyl-ω-tetranor-3,7-dithia-$PGE_1$ (ONO-AE1-329), was found to increase colony formation of HCA-7 cells to a similar extent as $PGE_2$ [26]. ONO-AE1-329 and $PGE_2$ but not $EP_2$ and $EP_3$ agonists, up-regulate the expression of c-fos and increased colony formation in a gallbladder adenocarcinoma cell line, Mz-ChA-2, in which COX-2 protein and mRNA are hardly detectable [53]. Furthermore, this activation of PI3K/Akt signaling by the $EP_4$ receptor induces functional expression of EGR-1 [40], a member of the zinc finger family of transcription factors which plays a key role in cell growth and differentiation by direct regulation of the expression of cyclin D1 [54].

While $PGE_2$ has been reported to inhibit the proliferation of certain colorectal cancer cell lines [55], the apparently anomalous effects could depend on characteristics of the cells such as different activation of prostanoid receptors and their cross talk or dependence on prostanoid stimulus. For instance, the growth of primary adult human keratinocytes is stimulated by activation of $EP_2$ receptors and is inhibited by activation of $EP_3$ receptors *via* an AC independent mechanism [56]. In a human colon cell line, an $EP_3$-selective agonist has been shown to inhibit cell growth of $EP_3$ receptor expressing but not of $EP_3$ receptor undetectable cells [29].

### B) Apoptosis

During carcinogenesis, apoptosis is decreased with substantial induction of antiapoptotic proteins. Several reports which addressed the possible causal linkage between expression of COX-2 and inhibition of apoptosis suggest that enzyme-promoted antiapoptosis is mediated by release of prostanoids, especially $PGE_2$. It has also been reported that the antiapoptotic protein, Bcl-2, is involved in antiapoptotic effects of COX-2 [57-59]. Furthermore, $PGE_2$ promotes induction of a cAMP-dependent cellular inhibitor of apoptosis,

c-IAP-2. Three cAMP agonists, $PGE_2$, cholera toxin and a membrane-permeable cAMP analog, 8-CPT-cAMP, all protect RIE-1, T84 and/or HCA-7 cells from Fas and staurosporine-induced apoptosis by induction of c-IAP-2 and delayed induction of LIVIN, another member of the IAP family [60]. PI3K/Akt is known to inhibit pro-apoptotic signaling through BAD, caspase-9 and Fas, while activating anti-apoptotic signaling through NFκB, an up-stream mediator of IAP [61].

### C) Inflammation and Immune Surveillance

In inflamed tissues, up-regulation of COX-2 and increased synthesis of $PGE_2$ (elevation 3-fold or more) can be observed. It is suggested that a high level of $PGE_2$ production in tumor tissue could mediate a profound alteration in the cytokine balance in the cancer microenvironment. For instance, $PGE_2$ may reduce tumor necrosis factor (TNF) production in lipopolysaccharide-treated murine macrophages [62]. Lung tumor-derived $PGE_2$ promotes induction of lymphocyte and macrophage IL-10, an immunosuppressive cytokine, while simultaneously inhibiting macrophage IL-12 production [63]. Furthermore, liver cells and macrophages isolated from $EP_4$ knockout mice have been documented to produce significantly less IL-1β and IL-6 than control samples [64].

Recent evidence suggests that change in expression of prostanoid receptors also correlates with several chronic inflammation diseases. $EP_4$-deficient mice, but not DP, $EP_1$, $EP_2$, $EP_3$, FP, IP, or TP deficient mice, develop severe dextran sodium sulfate-induced (DSS-induced) colitis with aggregation of neutrophils and lymphocytes in the colon. Also administration of AE3-208, an $EP_4$-selective antagonist, mimicks DSS-induced colitis in wild-type mice [65]. In a rheumatoid arthritis model, $EP_4$ receptor–deficient mice, but not their $EP_{1-3}$ counterparts, have shown decreased incidence and severity [64]. The apparently conflicting effects on inflammation reflect the complicated immune system and various functions of prostanoids. However, as immune suppression is well established to favor tumor growth, the facts provide a basis for a cause-and-effect link between chronic inflammation and carcinogenesis.

### D) Angiogenesis

Without vascular supply of nutrients and oxygen, tumors can not increase their mass. Hypoxia induces microvascular endothelial COX-2 expression [66] which in turn stimulates production of angiogenic factors. Several reports suggest that prostanoids can mediate tumor angiogenesis and, recently, $PGE_2$ was reported to mediate angiogenesis *via* stimulation of $EP_2$, $EP_4$ and TP receptors [67-69].

## CONCLUSIONS AND FUTURE DIRECTIONS

Increasingly, attention has become focused on studies of the significance of prostanoid receptors for carcinogenesis over the last several years. The present review aims to provide an up date of publications in this field of research, with particular attention to the possible mechanisms of prostanoid action and potential application of prostanoid receptor inhibitors/agonists for colon cancer prevention. Indeed, since COX-2 may be involved in cancer development in sites as widespread as the breast, stomach, head and neck, lung and pancreas [45], these might also be targets for chemoprevention by selective prostanoid receptor inhibitors. Further clarification of prostanoid receptor function is now a high priority and development of selective inhibitors needs to be further addressed.

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# Inhibition of intestinal carcinogenesis by a new flavone derivative, chafuroside, in oolong tea

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**A new flavone derivative, chafuroside, has been isolated as a strong anti-inflammatory compound from oolong tea leaves, and its structure determined to be (2*R*,3*S*,4*S*,4a*S*,11b*S*)-3,4,11-trihydroxy-2-(hydroxymethyl)-8-(4-hydroxyphenyl)-3,4,4a,11b-tetrahydro-2*H*,10*H*-pyrano[2′,3′:4,5]furo[3,2-g]chromen-10-one. To assess its potential to inhibit intestinal carcinogenesis, 2.5, 5 and 10 p.p.m. chafuroside was given in the diet to *Apc*-deficient Min mice for 14 weeks from 6 weeks of age. Total numbers of polyps were reduced to 83, 73 and 56% of the control value, respectively. Moreover, dietary administration at 10 and 20 p.p.m. reduced azoxymethane (AOM)-induced colon aberrant crypt foci (ACF) development in rats to 69% of the AOM-treated control value with the higher dose. Chafuroside-associated toxicity was not observed at 2.5–10 p.p.m. in Min mice and 10–20 p.p.m. in AOM-treated rats. These results suggest that chafuroside might be a good chemopreventive agent for colon cancer. (*Cancer Sci* 2006; 97: 248–251)**

Colon cancer is one of the most common cancers in developed countries[1] and epidemiological studies have shown that a Western-style diet, high in fat and red meat as well as low in fruits and vegetables, increases the risk.[2,3] Thus, foodstuff is a major focus for research, particularly with regard to identification of effective chemopreventive agents.

Epidemiological evidence suggests that drinking green tea (*Camellia sinensis*) is beneficial for cancer prevention.[4–6] Many animal studies also have shown that tea and its components have anticancer properties.[7,8] The major characteristic constituents of green tea are catechins, including EGCG.[9] In black tea, a large proportion of the catechins are converted into theaflavins and thearubigens through oxidation and polymerization. Another tea, oolong tea, is widely consumed in Asia, especially in China and Japan. The difference among green, black and oolong teas lies in fermentation: green tea is unfermented, black tea is completely fermented, and oolong tea is partially fermented.[7]

Recently, a strong anti-inflammatory compound named chafuroside, (2*R*,3*S*,4*S*,4a*S*,11b*S*)-3,4,11-trihydroxy-2-(hydroxymethyl)-8-(4-hydroxyphenyl)-3,4,4a,11b-tetrahydro-2*H*,10*H*-pyrano[2′,3′:4,5]furo[3,2-g]chromen-10-one, was isolated from oolong tea leaves with the aid of an inhibition test with DNFB-induced contact hypersensitivity in mice, and its total synthesis reported.[10,11] The compound was presumed to be produced during the partial fermentation process and showed strong anti-inflammatory activity in DNFB and 2,4,6-trinitro-1-chlorobenzene-induced contact hypersensitivity models.[10,11] Moreover, a preliminary study demonstrated the effective dose in the DNFB-induced contact hypersensitivity model to be approximately equal to that of indomethacin, a NSAID, which has been proven to have a cancer-chemopreventive influence.[12]

Although several reports have documented antioxidant, antiallergic and antiobesity activities of oolong tea extracts,[13–15] effects of individual constituents on colon carcinogenesis have hitherto not been described. In the present study, we therefore investigated the impact of chafuroside on intestinal polyp formation in *Apc*-deficient Min mice, an animal model of human familial adenomatous polyposis that develops numerous polyps in the intestinal tract.[16] We also investigated the impact of chafuroside on the formation of AOM-induced aberrant crypt foci, which are putative preneoplastic lesions in the F344 rat colon. In both cases chafuroside reduced the number of lesions, pointing to possible application as a chemopreventive agent for intestinal cancer.

## Materials and Methods

### Animals and chemicals

Female C57BL/6J-*Apc*$^{Min/+}$ mice (Min mice) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 5 weeks of age and genotyped using a method reported previously.[16] Heterozygotes, as well as wild-type (C57BL/6J) mice, were acclimated to laboratory conditions for 1 week, along with male F344 rats obtained from Charles River Japan (Atsugi, Japan) at 5 weeks of age. Three to five animals were housed per plastic cage, with sterilized softwood chips as bedding, in a barrier-sustained animal room, air-conditioned at 24 ± 2°C and 55% humidity, on a 12:12 h light:dark cycle. Food and water were available *ad libitum*. The animals were observed daily for clinical signs and mortality. Bodyweights and food consumption were measured weekly. The experiments were conducted according to the 'Guidelines for Animal Experiments in the National Cancer Center' of the Committee for Ethics of Animal Experimentation of the National Cancer Center.

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Abbreviations: AC, aberrant crypt; ACF, aberrant crypt foci; AOM, azoxymethane; COX, cyclooxygenase; DNFB, 2,4-dinitrofluorobenzene; EGCG, (–)-epigallocatechin-3-gallate; NSAID, non-steroidal anti-inflammatory drug.



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AOM was purchased from Sigma Chemical Co. (St Louis, MO, USA). Chafuroside was synthesized chemically at the University of Shizuoka (Shizuoka, Japan).[11] Its chemical structure is shown in Fig. 1. The purity of the compound was examined by $^1H$ nuclear magnetic resonance and high-performance liquid chromatography, and showed no concomitant peaks. The compound was pure enough, estimated to be above 99% (melting point of the compound was 229–232°C). Chafuroside concentrations of 2.5, 5, 10 and 20 p.p.m. were mixed into the powdered basal diet AIN-76 A (CLEA Japan, Tokyo, Japan) and confirmed to be stable in the diet under the experimental conditions used in the present study. The doses of chafuroside were selected according to our preliminary study in which chafuroside suppressed intestinal polyp formation in *Apc* gene-deficient mice.

### Intestinal polyp formation in Min mice

Female Min mice ($n = 9$–10/group) were fed diets containing 0 (control), 2.5, 5 or 10 p.p.m. chafuroside for 14 weeks from 6 weeks of age. All animals were anesthetized with ether before they were killed. The liver, kidneys and spleen were removed and weighed and the intestinal tract was resected, filled with 10% buffered formalin, and divided into four sections: three segments of small intestine: (1) proximal (4 cm in length from the pylorus ring of the stomach); (2) middle and (3) distal halves of the remainder; and (4) the colon. These segments were opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin. Polyp numbers and sizes, and their distributions in the intestine, were determined under a stereoscopic microscope.[17]

### AOM-induced ACF development in rats

Male F344 rats, 6 weeks of age, were treated subcutaneously with either AOM in sterile saline at a dose of 15 mg/kg bodyweight or with the saline vehicle, once a week for 2 weeks from 6 weeks of age. From 1 day before the first treatment with AOM, rats were fed control or experimental diets containing chafuroside at 10 or 20 p.p.m. for 4 weeks. At 10 weeks of age, they were killed under ether euthanasia and complete necropsies were carried out. The liver, kidneys and spleen were removed and weighed. The entire colon was resected, filled with 10% buffered formalin, opened longitudinally, and fixed flat between sheets of filter paper in 10% buffered formalin. The colon was then stained with 0.2% methylene blue in saline, and scored under a light microscope for the number of ACF per colon and the mean number of AC per focus.[18]

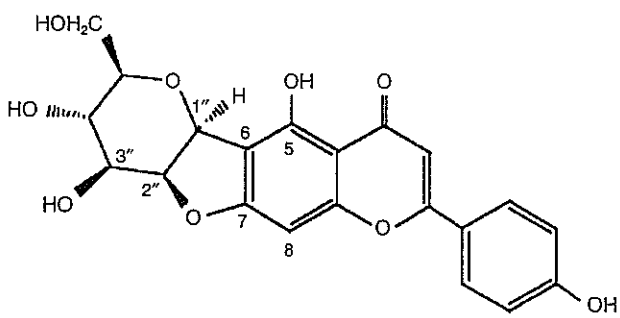


Fig. 1. Structure of chafuroside.

### Statistical analysis

The results were expressed as mean ± SD, and statistical analysis was carried out using Dunnett's multiple comparison test. In addition, the linear regression test was also used. Differences were considered to be statistically significant with $P$-values less than 0.05.

## Results

In Min mice, most polyps were located in the small intestine, with a preponderance in the distal parts, and only a few polyps were observed in the colons (Table 1). Treatment with chafuroside at 2.5, 5 and 10 p.p.m. for 14 weeks clearly decreased the total numbers of polyps to 83, 73 and 56% ($P < 0.01$) of the untreated control value, respectively (Table 1). The numbers of polyps in the proximal, middle and distal parts of small intestine in the 10 p.p.m. group were 54, 78 and 46% of the untreated control values, respectively (Table 1). Dose-dependent inhibition was observed in the number of polyps in the proximal ($r = -0.9958$, $P < 0.0005$) and distal parts ($r = -0.9129$, $P < 0.01$) of the small intestine, and in the total number of polyps ($r = -0.9863$, $P < 0.02$). As shown in Fig. 2, administration of chafuroside reduced the number of polyps mainly less than 1.0 mm in diameter. However, the number of polyps measuring ≥1.0 mm in diameter was not affected by chafuroside treatment. Survival rate, general conditions, food consumption and organ weights did not differ among the groups. No significant macroscopic changes were noted in the liver, kidney or spleen. Final body weights in the groups treated with 2.5, 5 and 10 p.p.m. were 103, 105 and 125% of the untreated control value, respectively.

In AOM-treated rats, administration of chafuroside at 10 and 20 p.p.m. in the diet for 4 weeks again did not affect

Table 1. Suppression of intestinal polyp development in Min mice by chafuloside, shown by the number of polyps per mouse

| Group (p.p.m) | No. mice | Small intestine | | | Colon | Total |
|---|---|---|---|---|---|---|
| | | Proximal | Middle | Distal | | |
| 0 | 9 | 17.7 ± 9.8 | 39.7 ± 11.6 | 86.8 ± 27.1 | 0.78 ± 0.67 | 144.9 ± 37.7 |
| 2.5 | 7 | 15.3 ± 7.0 (86) | 34.4 ± 7.7 (87) | 70.0 ± 9.9 (81) | 0.86 ± 0.69 (110) | 120.6 ± 21.1 (83) |
| 5.0 | 9 | 13.0 ± 4.3 (73) | 33.4 ± 8.6 (84) | 59.2 ± 18.9 (68)* | 0.56 ± 0.53 (72) | 106.2 ± 26.5 (73) |
| 10.0 | 8 | 9.6 ± 3.1 (54)* | 31.0 ± 19.9 (78) | 40.0 ± 21.8 (46)** | 0.75 ± 0.71 (96) | 81.4 ± 41.9 (56)** |

Data are mean ± SD. Numbers in parentheses are percentages of the control basal diet values. *Significantly different from the basal diet group at $P < 0.05$. **Significantly different from the basal diet group at $P < 0.01$.

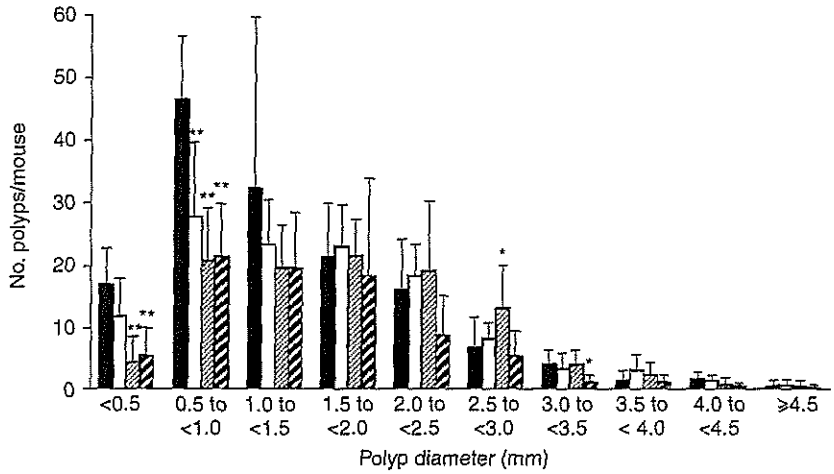


Fig. 2. Effects of chafuroside on the size distribution of intestinal polyps in Min mice. Min mice were fed a basal diet (■) or a diet containing 2.5 (□), 5 (▨) or 10 p.p.m. (▨) chafuroside for 14 weeks. The number of polyps/mouse in each size class is given as a mean value (bars represent SD). *$P < 0.05$, **$P < 0.01$.

general conditions, body weights, food consumption or organ weights. No significant macroscopic changes were observed in the liver, kidney or spleen. ACF were observed in all rats treated with AOM, mainly located in the distal colon. Administration of 10 and 20 p.p.m. chafuroside reduced the total numbers of ACF per colon to 79 ($P < 0.05$) and 69% ($P < 0.01$) of the AOM-treated control value, respectively (Table 2). The total number of AC per colon was also decreased by 16 and 30%, respectively (Table 2). However, treatment with chafuroside did not decrease the mean number of AC per focus (Table 2).

## Discussion

In the present study, we obtained clear evidence that a new flavone derivative, chafuroside, suppresses development of intestinal polyps in Min mice and AOM-induced colon ACF in F344 rats at doses of 5–20 p.p.m. in the diet. Although it is a natural compound found in tea leaves, the doses effective in Min mice were much lower than those reported earlier for well-known, naturally occurring and synthesized chemopreventive agents. Indeed, the effective dose to reduce numbers of polyps in Min mice was 10 p.p.m. for chafuroside. This value is lower than with (+)-catechin at 1000 p.p.m.,[19] genistein at 1000 p.p.m.,[20] curcumin at 2000 p.p.m.,[21] and with synthesized aspirin at 250 p.p.m.,[22] piroxicam at 200 p.p.m.[23] and celecoxib at 1500 p.p.m.[24] As treatment with 2.5–10 p.p.m. chafuroside affected only the polyps of smaller size, it might be important to clarify the mechanism by which chafuroside inhibits polyp growth. It has been reported that 100 p.p.m. EGCG is effective for approximately 60% inhibition of AOM-induced ACF formation in F344 rats,[25] and our results thus suggest that chafuroside possesses a strong potential to inhibit development of putative preneoplastic lesions in the colon.

Because there were no signs of chafuroside-induced adverse effects in the present study, long-term consumption for cancer prevention in humans is conceivable. The daily estimated consumption level from a diet containing 10 p.p.m. chafuroside in mice corresponds to approximately 120 mg per day for a 60-kg adult man. The average concentration of chafuroside in a commercially available oolong tea in Japan is almost 55 μg/L (unpublished data). An adult human might reach an effective dose of chafuroside with consumption of more than 100 L of oolong tea. Therefore, taking a chafuroside supplement or drinking concentrated oolong tea may be useful for cancer prevention.

The strong anti-inflammatory and chemopreventive effects are presumably related to the two characteristic moieties of chafuroside: the mannose moiety of which the C1 position provides a C-glycoside linkage with the C6 position of apigenin, and the dihydrofuran moiety, obtained

Table 2. Effects of chafuroside on azoxymethane (AOM)-induced aberrant crypt focus (ACF) formation in F344 rats

| Group | No. rats with ACF | Total no. ACF/colon (%) | Total no. AC/colon (%) | Mean no. AC/focus |
|---|---|---|---|---|
| AOM treatment | | | | |
| Control diet | 9/9 | 278 ± 51 | 618 ± 81 | 2.25 ± 0.18 |
| Chafuroside (10 p.p.m.) | 9/9 | 219 ± 42 (79)* | 522 ± 105 (84) | 2.40 ± 0.27 |
| Chafuroside (20 p.p.m.) | 9/9 | 192 ± 42 (69)** | 435 ± 69 (70)** | 2.29 ± 0.30 |
| Saline treatment | | | | |
| Control diet | 0/3 | 0 | 0 | 0 |
| Chafuroside (10 p.p.m.) | 0/3 | 0 | 0 | 0 |
| Chafuroside (20 p.p.m.) | 0/3 | 0 | 0 | 0 |

Data are mean ± SD. Numbers in parentheses are percentages of the control basal diet values. *Significantly different from the basal diet group at $P < 0.05$. **Significantly different from the basal diet group at $P < 0.01$. AC, aberrant crypt.



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